Supplemental data

Supplemental Materials and Methods

Protein Purification

The mature form of Hsc20 (residues 10-184) was purified from *E. coli* C41(DE3) cells harboring pET21d Hsc20 plasmid, encoding Hsc20 with a polyhistidine tag at the C-terminus. Expression was induced with 1 mM isopropyl 1-thio-D-glucopyranoside at A_{600} = 0.6 and cells were grown in LB media at 37°C for 3 h. Cells were harvested and lysed by French press in buffer F (20 mM Tris-HCl pH 8.0, 10% (v/v) glycerol, 500 mM NaCl, 1 mM phenylmethylsulphonyl fluoride, 30 mM imidazole). After a clarifying spin, the supernatant was supplemented with ammonium sulfate to 0.35 g/mL. After 1 h of incubation at 4°C the solution was centrifuged. The pellet was resuspended with buffer F and dialyzed against buffer F (12 h). The sample was loaded on Ni-NTA resin equilibrated with buffer F and washed with buffer F, buffer F with 1 mM ATP, 2 mM $MgCl_2$, 1 M NaCl and again with buffer F. Hsc20 was eluted with a 30-300 mM imidazole gradient in buffer F (12 column volumes). Fractions containing Hsc20 were concentrated by ultrafiltration and dialyzed (4 h) against buffer G (20 mM Tris-HCl 8.0, 10% (v/v) glycerol, 50 mM NaCl, 5 mM β-mercaptoethanol) before loading on Q-Sepharose resin equilibrated with buffer G. After loading, the Q-Sepharose resin was washed with buffer G, and Hsc20 was eluted with a 50-300 mM NaCl gradient in buffer G (10 column volumes). Fractions containing Hsc20 were concentrated by ultrafiltration and dialyzed (4 h) against buffer G, frozen in liquid nitrogen and stored at -70°C.

The mature form of Mge1 (residues 45-228) with a C-terminal polyhistidine tag was purified from *E. coli* strain BL21(DE3) harboring the pET21dScMGE1 plasmid. Expression was induced by addition of 1 mM isopropyl 1-thio-D-glucopyranoside in LB media at A_{600} = 0.6. After 3 h at 37°C, cells were harvested and lysed in a French press in buffer H (25 mM Tris 8.0, 150 mM NaCl, 10% (v/v) glycerol, 1 mM phenylmethylsulphonyl fluoride). After a clarifying spin, the supernatant was loaded on a Ni-NTA column equilibrated in buffer H. After loading the Ni-NTA resin was washed with buffer H with 500 mM NaCl. Proteins were eluted with a 10–500 mM imidazole gradient in buffer H (16 column volumes). Fractions containing Mge1 were collected and dialyzed against buffer J (50mM Tris pH 7.4, 10% (v/v) glycerol, 25mM NaCl) and stored at -70°C.

Ssq1* with a N-terminal GST tag (Ssq1*^{GST}) was purified from *E. coli* strain BL21-CodonPlus (DE3) harboring the pRSFDuet1-SSQ1T239AGST/HEP1 plasmid. Expression was induced by addition of 1 mM isopropyl 1-thio-D-glucopyranoside in TB media at A_{600} = 0.6. After 3 h at 30°C, cells were harvested and lysed in a French press in buffer L (25 mM Tris 8.0, 200 mM NaCl, 10% (v/v) glycerol, 1 mM dithiothreitol, 0.05% Triton X-100, 1 mM phenylmethylsulphonyl fluoride). After a clarifying spin, the supernatant was loaded on an Agarose CL-4B resin with immobilized reduced glutathione equilibrated with buffer L. After loading the Agarose resin was washed with buffer L without 1 mM phenylmethylsulphonyl fluoride, buffer L with 1 mM ATP and 10 mM $MgCl_2$ and again with buffer L. Proteins were eluted with buffer L with 50 mM reduced glutathione (10 column volumes). Fractions containing Ssq1*^{GST} were collected and dialyzed against buffer M (50 mM Tris pH 8.0, 10% (v/v) glycerol, 50 mM NaCl, 5 mM β-mercaptoethanol) and loaded on a Q-Sepharose column equilibrated with buffer M. After loading the resin was washed with buffer M. Proteins were eluted with a 50–300

mM NaCl gradient in buffer M (16 column volumes). Fractions containing Ssq1*^{GST} were concentrated by ultrafiltration and dialyzed (4 h) against buffer N (20 mM KPi pH 6.8; 150 mM NaCl; 10% (v/v) glycerol; 0.05% Triton X-100; 5 mM β-mercaptoethanol). Then sample was loaded on hydroxyapatite (HTP) resin equilibrated with buffer N. After loading the HTP resin was washed with buffer N, and Ssq1*^{GST} was eluted with buffer N with a 20-220 mM KPi gradient in buffer N. Fractions containing Ssq1*^{GST} were concentrated by ultrafiltration and dialyzed (4 h) against buffer O (20 mM HEPES-KOH pH 8.0; 100 mM KCl; 10% (v/v) glycerol; 5 mM β–mercaptoethanol), frozen in liquid nitrogen and stored at -70°C.

Hsc20 with a C-terminal GST tag (Hsc20^{GST}) was purified from *E. coli* strain C41(DE3) harboring the pET3a-HSC20-GST plasmid. Expression was induced by addition of 1 mM isopropyl 1-thio-D-glucopyranoside in LB media at A_{600} = 0.6. After 4 h at 30°C, cells were harvested and lysed in a French press in buffer P (25 mM Tris 8.0, 200 mM NaCl, 10% (v/v) glycerol, 1 mM dithiothreitol, 0.05% Triton X-100, 1 mM phenylmethylsulphonyl fluoride). After a clarifying spin, the supernatant was loaded on Agarose CL-4B resin with immobilized reduced glutathione equilibrated in buffer P. After loading the Agarose resin was washed with buffer P without 1 mM phenylmethylsulphonyl fluoride, buffer P with 1 mM ATP and 10 mM $MgCl_2$ and again with buffer P. Proteins were eluted with buffer P with 50 mM reduced glutathione (10 column volumes). Fractions containing Hsc20^{GST} were concentrated by ultrafiltration and dialyzed (4 h) against buffer R (20 mM Tris-HCl pH 8.0; 50 mM NaCl; 10% (v/v) glycerol), frozen in liquid nitrogen and stored at -70°C.

Ssq1 ATPase Assay

Ssq1 ATPase activity was measured as described in [1] using an enzyme-coupled spectrophotometric assay [2]. Reaction mixtures contained 1 μM Ssq1 or Ssq1* alone or in the presence of 10 μM Isu1, 1 μM Hsc20 and 1 μM Mge1, in buffer K (50 mM HEPES-KOH, pH 7.5, 150 mM KCl, 20 mM magnesium acetate, 1 mM dithiothreitol, 0.265 mM NADH, 100 U/mL lactic dehydrogenase, 70 U/mL pyruvate kinase, 2.8 mM phosphoenolpyruvate). Reactions (500 μL) were initiated by the addition of ATP to 1 mM concentration. Incubation was carried out at 25°C. NADH absorbance was measured at 340 nm for 500 s in a JASCO V-660 UV-Vis spectrophotometer.

Densitometry analysis

Densitometry quantifications of Coomassie Blue stained gels were performed using the program ImageJ [3]. The values obtained for each analyzed protein were normalized to the levels of bait protein (e.g., Isu1^{GST} or GST) in each analyzed lane. The background binding to GST signals were subtracted from the normalized data. For the quantification of Ssq1* and Hsc20 bound to Isu1^{GST} in the Figure 1(B), the densitometric signal for either Ssq1* or Hsc20 bands in lane without Hsc20 was subtracted from signals obtained in the presence of Hsc20. Then the signals were normalized to the signal obtained for Isu1^{GST} in each lane.

Structural models of Isu1 and Hsc20-Isu1 complexes

Structural model of Isu1 was prepared with I-TASSER [4] using as a template the crystal structure of bacterial IscU in complex with IscS (PDB ID: 3LVL), with C-score equal 1.29. The model was placed in 8 x 8 x 8 nm dodecahedron box and solvated with ~13000 water molecules. The system was energy-minimized, and the protein side chains were relaxed in 50 ns-long MD simulation with restrained protein backbone atoms. The system was then simulated in equilibrium MD, using CHARMM36 and AMBER99SB-

ILDN [5] forcefields, for 1 μs. The resulting trajectories were clustered using gmx cluster tool from GROMACS package with 0.3 nm Cα RMSD cutoff, and central structures of top 7 clusters representing distinct conformational states were used for docking to Hsc20.

To obtain the structural model of Hsc20-Isu1 complex, the structures of Isu1 were docked to 8 central structures of Hsc20, derived from equilibrium MD simulations of the crystal structure (PDB ID: 3UO3), as described in [6], using ClusPro [7] without restraints on protein–protein interactions. From the 560 structures obtained from docking we selected 56 that had Isu1 located near Hsc20 CTD and 56 that had the lowest energy estimate. Redundancy among these models was removed using gmx cluster tool from GROMACS package, with single-linkage clustering method and 0.5 nm RMSD cutoff on Isu1 position, resulting in a model of the Hsc20-Isu1 complex. The Hsc20-Isu1 complex was then placed in 12 x 12 x 12 nm dodecahedron box, solvated with ~37000 water molecules and energy minimized. The system was then simulated in equilibrium MD for 3 μs, using GROMACS 5.0.4, with temperature kept at 300 K. Trajectories were superimposed on Cα atoms of Hsc20 and clustered based on backbone of Isu1, using gmx cluster with Jarvis-Patrick method and 0.5 nm RMSD cutoff. The central structure of a dominant binding mode was used for further analysis as a representative model of Hsc20-Isu1 complex.

Protein identification with mass spectrometry

Peak fractions from SEC were separated on SDS-PAGE (Bolt™ 12%, Bis-Tris, 1.0 mm, Mini Protein Gels, Invitrogen) and processed as described in [8]. Briefly, excised gel pieces containing Ssq1*, Hsc20 and Isu1 were de-stained and subjected to in-gel reduction using DTT, alkylation using iodoacetamide, and overnight trypsin digestion. Peptides were then extracted from gel, and the samples were dried with a SpeedVac, dissolved in 1% acetic acid in water, and desalted using the StageTips C18 column (Thermo Scientific).

LC/MS–MS analysis was performed in the Mass Spectrometry Laboratory (Intercollegiate Faculty of Biotechnology, University of Gdansk) using an Ekspert MicroLC 200 Plus system (Eksigent) equipped with ChromXP C18CL column (3 μm, 120 Å, 150×0.3 mm) and coupled with hybrid TripleTOF 5600+ mass spectrometer with DuoSpray Ion Source (AB SCIEX), controlled by SCIEX Analyst TF 1.7.1 software. All experiments were performed in a positive ion mode. Peptides were separated by a 28 min, 20-98% gradient of acetonitrile in 0.1% (v/v) formic acid, with flow rate of 5 μL/min, followed by 2 min regeneration and re-equilibration. Each cycle of applied DDA method comprised of precursor spectra accumulation in 100 ms in the range of 400-1200 m/z, followed by top 20 candidate ions per scan in 50 ms in the range of 100-1800 m/z resulting in a total cycle time of 1.15 s.

Data were searched with PEAKS Studio 11 software [9] against the SwissProt *E. coli* database (taxonomy ID: 562, retrieval date: 15th February 2023) plus the sequences of Ssq1, Hsc20, Isu1. Parent and fragment mass tolerances were set to 25 ppm and 0.05 Da, respectively, and tryptic peptides with maximum of three missed cleavages were included. Cysteine carbamidomethylation was specified as a fixed modification, while N-terminal acetylation and methionine oxidation were specified as variable modifications. Protein identification was validated using a target-decoy search strategy with 1% FDR threshold.

Native mass spectrometry

Samples containing Ssq1*-Hsc20-Isu1 and Hsc20-Isu1 complexes were desalted and buffer-exchanged using P-6 Micro BioSpin columns (Bio-Rad) pre-equilibrated with 100 mM ammonium acetate. Samples were introduced into the SYNAPT G2-HDMS mass spectrometer (Waters) by nano-electrospray ionization using gold-coated glass capillaries. MS settings were adjusted as follows: 1.4–1.6 kV capillary voltage, 25–75 V sampling cone, 3 V extractor cone, 30 °C source temperature, 15–30 V trap collision energy, and 5 V transfer collision energy. Pressures throughout the instrument were 6–9 mbar backing, 3.1 mbar in the ion mobility cell, and 2.5 10−2 mbar for the trap and transfer cell, respectively. Mass spectra were processed in MassLynx v4.1 (Waters) and deconvoluted using UniDec 5.2 [10].

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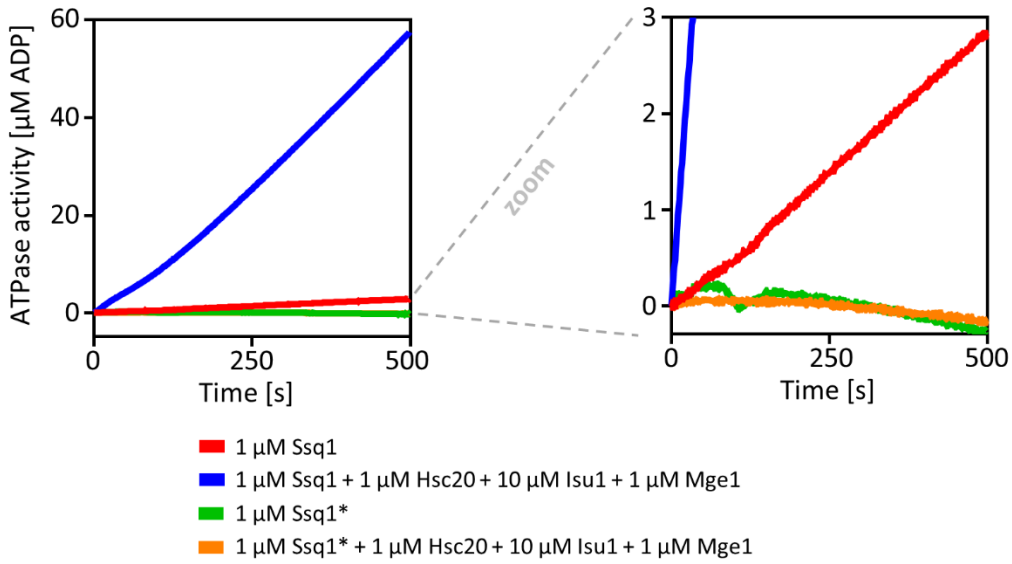


Figure S1. ATP hydrolysis by Ssq1* and Ssq1.
ATP hydrolysis was measured at 1 mM ATP using an enzyme coupled assay for Ssq1 and Ssq1* both alone and in the presence of Hsc20, Isu1 and a nucleotide exchange factor Mge1 at indicated concentrations.

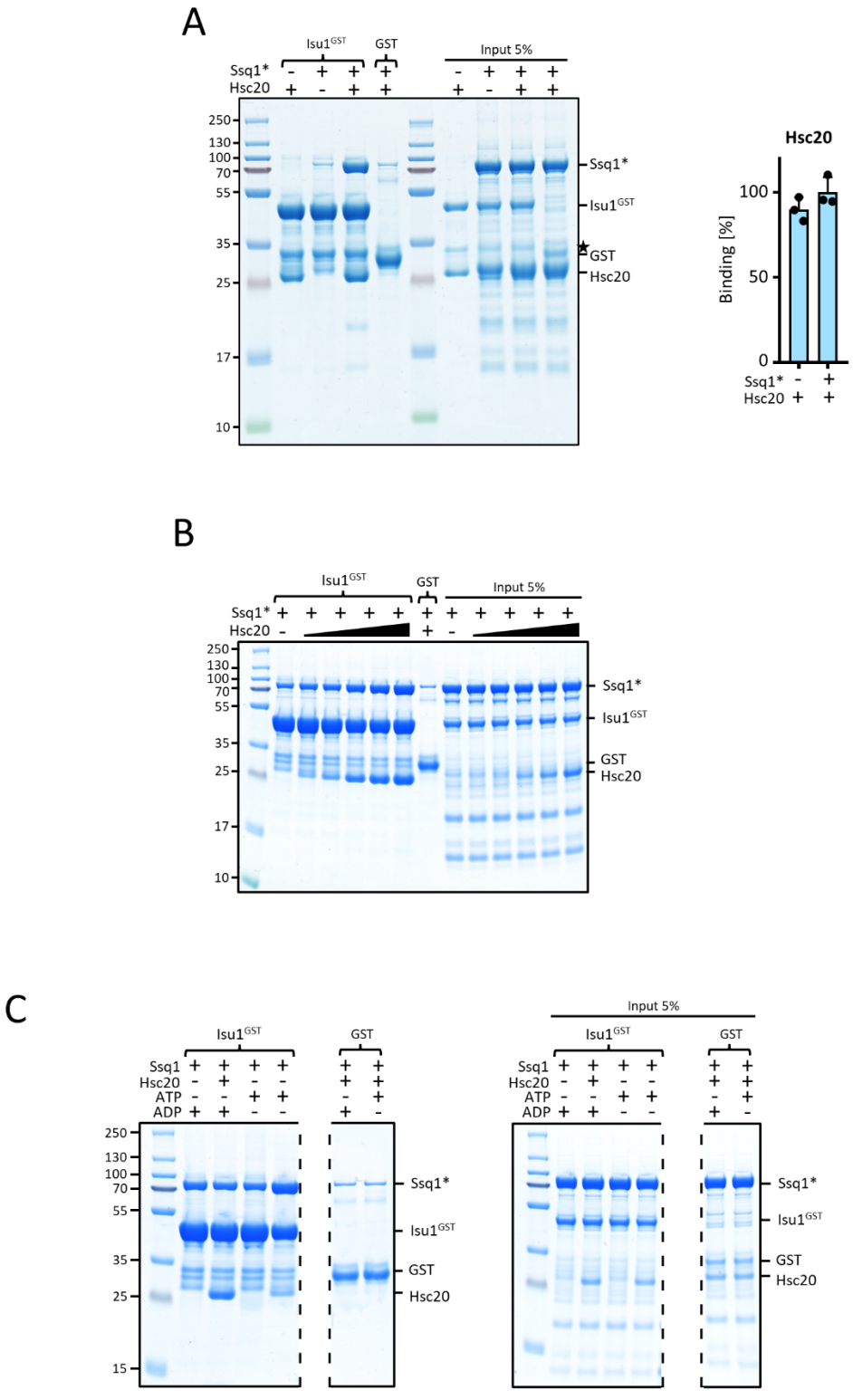


Figure S2. SDS-PAGE gels for Figure 1(B) and pull down of Ssq1 in the presence of ATP and ADP.

(A) (left) The entire gel for pulldown experiment presented in Figure 1(B) (left). Isu1^{GST} or GST at 2.5 μM was incubated with Ssq1* (5 μM) and Hsc20 (2.5 μM), as indicated. Glutathione resin was

added to pull-down GST and associated proteins, which were then separated by SDS-PAGE and stained with Coomassie blue. “Input 5%” - 5% loading control; ★ - marks the $Isu1^{GST}$ degradation product. (right) Quantification of Hsc20 in three independent experiments. The amount of pulled Hsc20 was quantified by densitometry and corrected for background binding to GST alone; Hsc20 level for $Isu1^{GST}$ interacting with Hsc20 and Ssq1* was set at 100%. Error bars represent SD.

(B) The entire gel for Hsc20 titration from Figure 1(B) (right). $Isu1^{GST}$ (2.5 μM) was incubated with Ssq1* (5 μM) and increasing concentration of Hsc20: 0.0, 0.25, 0.5, 1.5, 2.5, 5.0 μM. Reaction mixtures were treated as described in (A). Background binding to GST alone (GST) is shown for 5.0 μM Hsc20. “Input 5%” – 5% loading control.

(C) (left) $Isu1^{GST}$ (2.5 μM) was incubated with Ssq1 and Hsc20 (5 μM and 2.5 μM, respectively, as indicated) in the presence of ATP or ADP at 2 mM. Glutathione resin was added to pull-down GST and associated proteins, which were then separated by SDS-PAGE and stained with Coomassie blue. (**right) “**Input 5%” – 5% loading control.

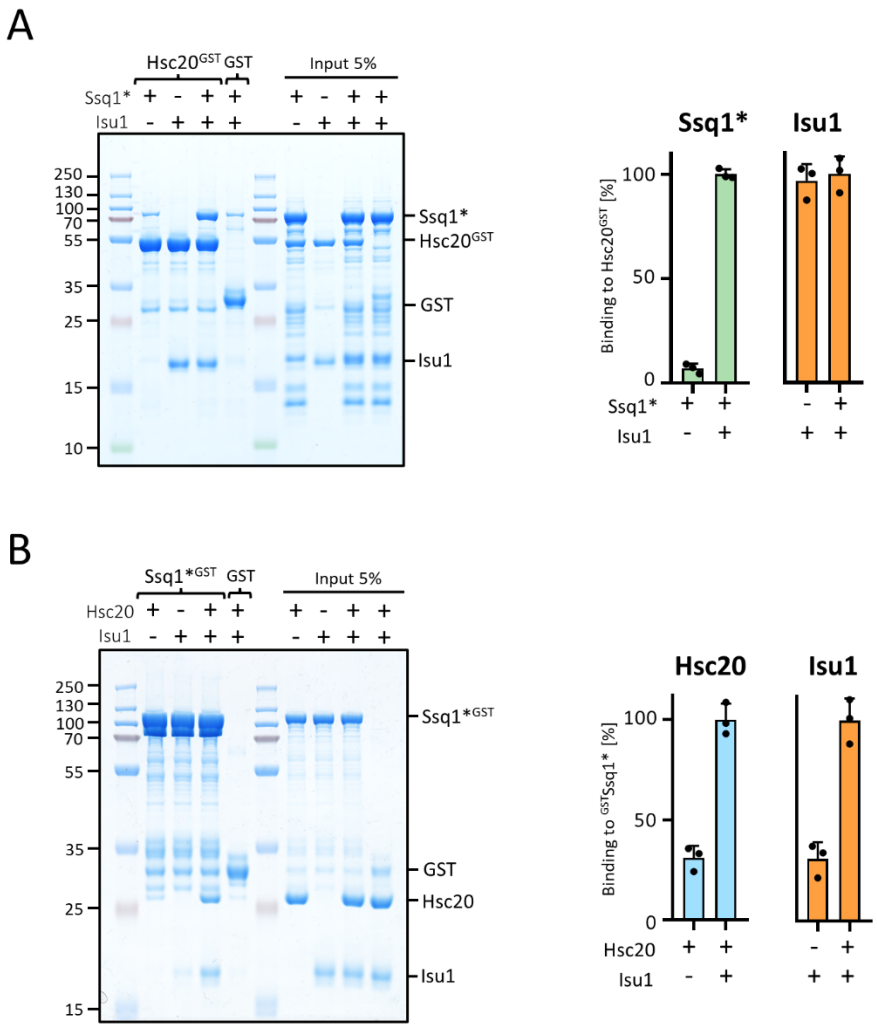


Figure S3. Pull down of the tripartite complex using Hsc20^{GST} and Ssq1*^{GST}.
(A) (left). Hsc20^{GST} or GST at 2.5 μM was incubated with Ssq1* (5 μM) and Hsc20 (2.5 μM), as indicated. Glutathione resin was added to pull-down GST and associated proteins, which were then separated by SDS-PAGE and stained with Coomassie blue. “Input 5%” - 5% loading control. (right) Quantification of Ssq1* and Isu1 in three independent experiments. The amounts of pulled down Ssq1* and Isu1 were quantified by densitometry and corrected for background binding to GST alone; Ssq1* and Isu1 levels for Hsc20^{GST} interacting with Ssq1* and Isu1 were set at 100%. Error bars represent SD.
(B) (left). Ssq1*^{GST} or GST at 2.5 μM was incubated with Hsc20 (2.5 μM) and Isu1 (2.5 μM), as indicated. Glutathione resin was added to pull-down GST and associated proteins, which were then separated by SDS-PAGE and stained with Coomassie blue. “Input 5%” - 5% loading control. (right) Quantification of Hsc20 and Isu1 in three independent experiments. The amounts of pulled down Hsc20 and Isu1 were quantified by densitometry and corrected for background binding to GST alone; Hsc20 and Isu1 levels for Ssq1*^{GST} interacting with Hsc20 and Isu1 were set at 100%. Error bars represent SD.

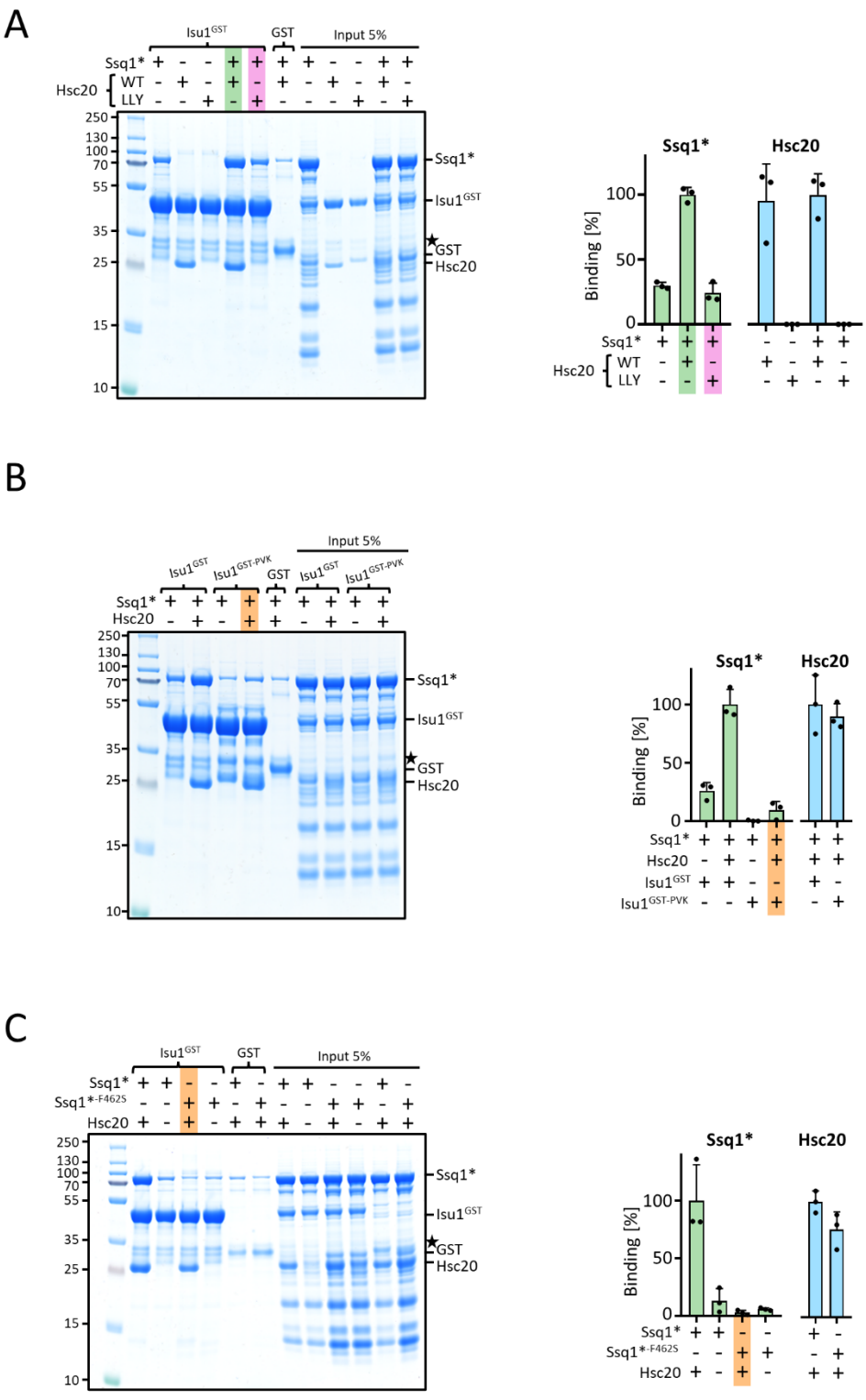


Figure S4. SDS-PAGE gels and quantification of Ssq1* and Hsc20 for Figure 1(C).
(A) SDS-PAGE gel and quantification of Ssq1* and Hsc20 for Hsc20^{LLY} pulldown from Figure 1(C). (left) Isu1^{GST} or GST (2.5 μM) was incubated with Ssq1* (5 μM) and Hsc20 wild-type (WT) or L105A, L106A, Y109A (LLY) variant, defective in interaction with Isu1, (2.5 μM). Glutathione

resin was added to pull-down GST and associated proteins, which were then separated by SDS-PAGE and stained with Coomassie blue. (right) Quantification of Ssq1* and Hsc20 in three independent experiments. The amounts of pulled down protein were quantified by densitometry and corrected for background binding to GST alone; protein levels for $Isu1^{GST}$ interacting with Ssq1* and Hsc20 WT were set at 100%. Quantifications of Ssq1 used in Figure 1(C) are marked by colored boxes. “Input 5%” - 5% loading control. ★ - marks the $Isu1^{GST}$ degradation product. Error bars represent SD.

(B) SDS-PAGE gel and quantification of Ssq1* and Hsc20 for $Isu1^{GST\text{-}PVK}$ pulldown from Figure 1(C). (left) $Isu1^{GST}$ or $Isu1^{GST\text{-}PVK}$ (P134A, V135A, K136A - variant defective in Ssq1 binding) or GST alone (2.5 μM) was incubated with Ssq1* (5 μM) and Hsc20 (2.5 μM), as indicated. Reaction mixtures were treated as described in (A). (right) Quantification of Ssq1* and Hsc20 in three independent experiments as described in (A); protein levels for $Isu1^{GST}$ interacting with Ssq1* and Hsc20 WT were set at 100%. Quantifications of Ssq1 used in Figure 1(C) is marked by orange box. “Input 5%” - 5% loading control. ★ - marks the $Isu1^{GST}$ degradation product. Error bars represent SD.

(C) SDS-PAGE gel and quantification of Ssq1* and Hsc20 for $Ssq1^{*\text{-}F462S}$ pulldown from Figure 1(C). (left) $Isu1^{GST}$ or GST alone (2.5 μM) was incubated with Ssq1* or $Ssq1^{*\text{-}F426S}$ variant, defective in interaction with Isu1 (5 μM) and Hsc20 (2.5 μM). Reaction mixtures were treated as described in (A). (right) Quantification of Ssq1* and Hsc20 in three independent experiments as described in (A). Protein levels for Isu1GST interacting with Ssq1* and Hsc20 WT were set at 100%. Quantification of Ssq1 used in Fig. 1C is marked by orange box.
“Input 5%” - 5% loading control. ★ - marks the $Isu1^{GST}$ degradation product. Error bars represent SD.

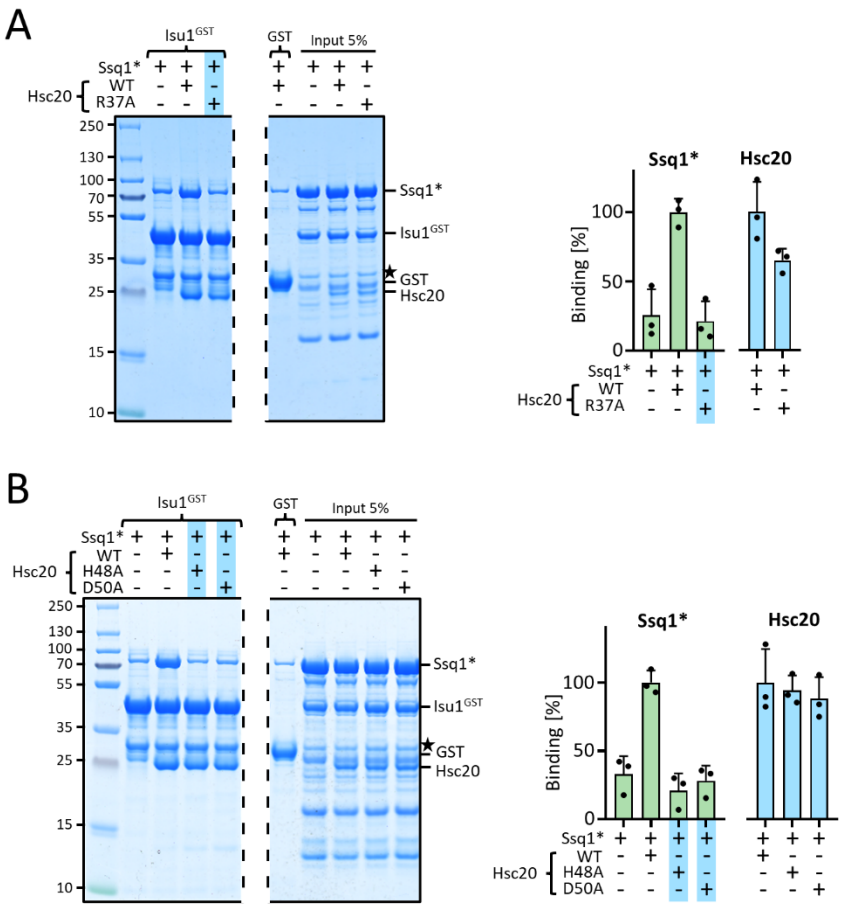


Figure S5. SDS-PAGE gels and quantifications of Ssq1* and Hsc20 for Figure 1(C).
(A) SDS-PAGE gel and quantification of Ssq1* and Hsc20 for Hsc20^{R37A} pulldown from Figure 1(C). (left) Isu1^{GST} or GST alone (2.5 μM) was incubated with Ssq1* (5 μM) and Hsc20 wild-type (WT) or J-domain variant R37A, defective in interaction with Ssq1 (2.5 μM). Glutathione resin was added to pull-down GST and associated proteins, which were then separated by SDS-PAGE and stained with Coomassie blue. Dashed lines indicate part of the same gel where irrelevant samples were cropped out of the image. (right) Quantification of Ssq1* and Hsc20 for three independent experiments. The amounts of pulled down protein were quantified by densitometry and corrected for background binding to GST alone; protein levels for Isu1^{GST} interacting with Ssq1* and Hsc20 WT were set at 100%. Quantification of Ssq1 used in Figure 1(C) is marked by light blue box. “Input 5%” - 5% loading control; ★ - marks the Isu1^{GST} degradation product. Error bars represent SD.
(B) SDS-PAGE gel and quantification of Ssq1* and Hsc20 for Hsc20^{H48A} and Hsc20^{D50A} pulldowns from Figure 1(C). (left) Isu1^{GST} or GST alone (2.5 μM) was incubated with Ssq1* (5 μM) and Hsc20 wild-type (WT) or HPD loop variants: H48A or D50A, defective in interaction with Ssq1 (2.5 μM). Reaction mixtures were treated as described in (A). Dashed lines indicate part of the same gel where irrelevant samples were cropped out of the image. (right) Quantification of Ssq1* and Hsc20 for three independent experiments as described in (A). Protein levels for Isu1^{GST} interacting with Ssq1* and Hsc20 WT were set at 100%. Quantifications of Ssq1 used in Figure 1(C) are marked by light blue boxes.
“Input 5%” - 5% loading control; ★ - marks the Isu1^{GST} degradation product. Error bars represent SD.

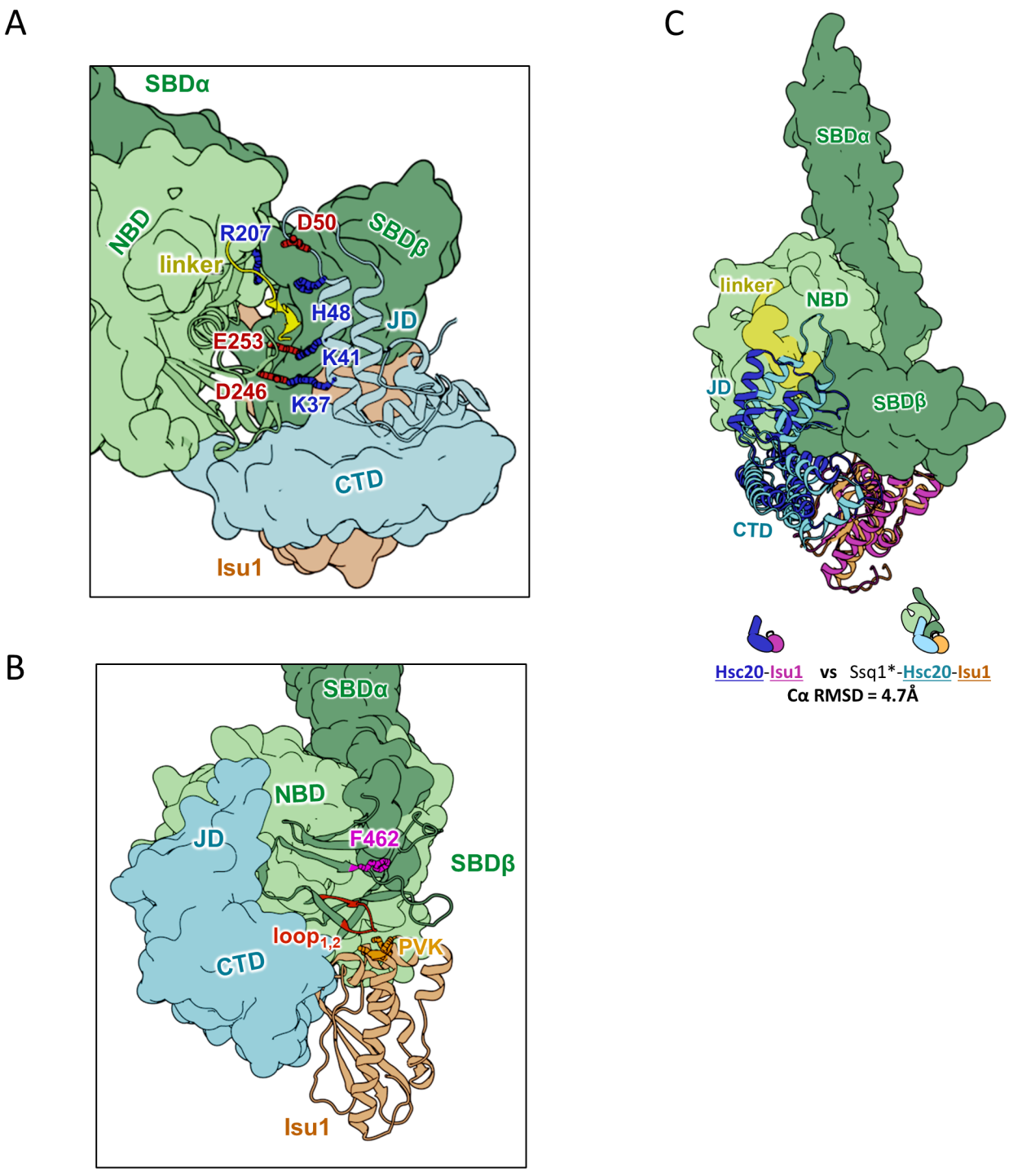
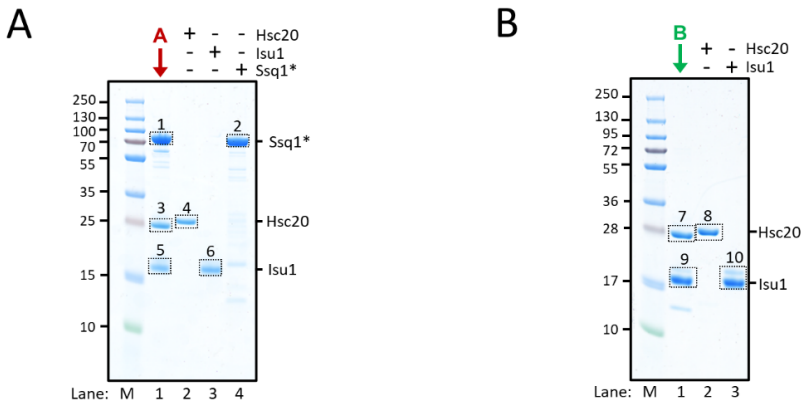


Figure S6. Structural model of the tripartite Ssq1(ATP)-Hsc20-Isu1 complex.
(A-B) Zoom in on J-domain (JD) – Ssq1(ATP) binding interface and LPPVK of Isu1 interaction with SBDβ of Ssq1. Hsc20 – light blue, Isu1- brown, Ssq1: NBD – light green, SBDβ dark green, interdomain linker- yellow. **(A)** JD residues (K37, K41, H48, D50) interacting with Ssq1 residues (D246, E253, R207) are indicated. **(B)** LPPVK loop of Isu1 is close to loop 1,2 of the substrate binding cleft of SBDβ.

(C) Hsc20 and Isu1 are arranged very similarly in both the tripartite Ssq1(ATP)-Hsc20-Isu1 and bipartite Hsc20-Isu1 complexes. Structural alignment of the bipartite Hsc20-Isu1 (see Supplemental Methods for how model of this complex was obtained) and tripartite Ssq1(ATP)-Hsc20-Isu1 complexes. The Cα root mean square deviation (RMSD) is 4.7 Å. The Hsc20 and Isu1 are represented in dark blue/light blue, violet/orange in the bipartite and tripartite complexes, respectively.



C

Sample	Protein	-10lgP	Coverage	Unique peptides
1	Ssq1	496.77	53.10%	36
2	Ssq1	547.23	60.68%	38
3	Hsc20	253.74	48.59%	8
4	Hsc20	115.87	31.64%	5
5	Isu1	262.07	49.28%	6
6	Isu1	189.67	37.68%	4
7	Hsc20	294.18	48.59%	8
8	Hsc20	254.66	72.32%	10
9	Isu1	200.69	49.28%	7
10	Isu1	226.61	50.72%	8

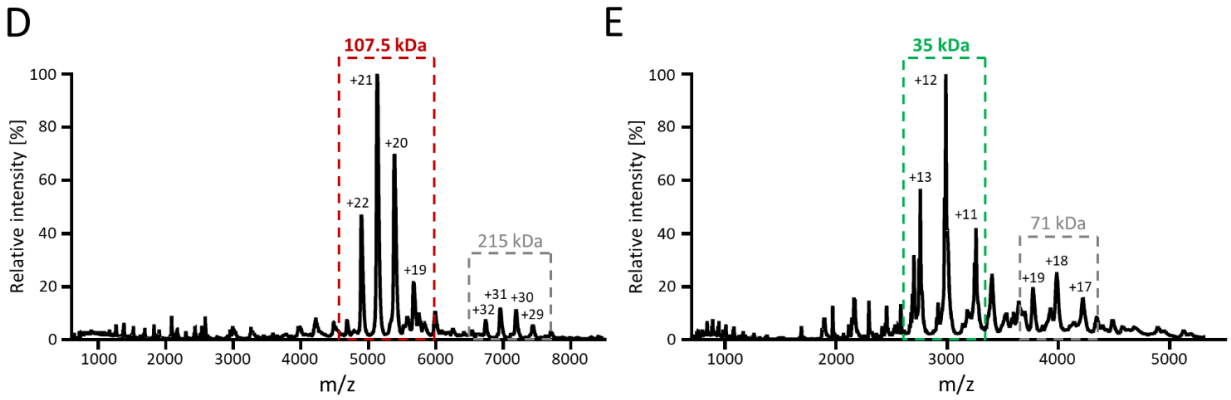


Figure S7. Identification of proteins and native MS spectra for Figure 2.
(A, B) Fractions representing peak-A **(A)** and peak-B **(B)** were separated by SDS-PAGE along with purified Ssq1, Hsc20 and Isu1 proteins, as indicated. Note that Hsc20 in the tripartite complex that has no tag and migrates faster than purified Hsc20 with a C-terminal His tag.
(C) Mass-spectrometry identification of proteins in peak-A and peak-B. Positions of bands analyzed by mass spectrometry (MS) are indicated in (A) and (B). Identification of peptides detected in LC-MS/MS analysis of the excised bands was performed in PEAKS Studio 10.6 software, using PEAKS standard protocol. PEAKS significance score (-10lgP), coverage of protein sequences with peptides, and number of unique peptides detected are indicated.
(D, E) Native mass spectrometry of purified tripartite Ssq1*-Hsc20-Isu1 and bipartite Hsc20-Isu1 complexes. Native mass spectra of the tripartite (D – red frame) and bipartite (E- green frame)

complexes. Minor peaks (gray frames) most likely represent dimers of the tripartite and bipartite complexes. Predicted molecular weights are indicated.

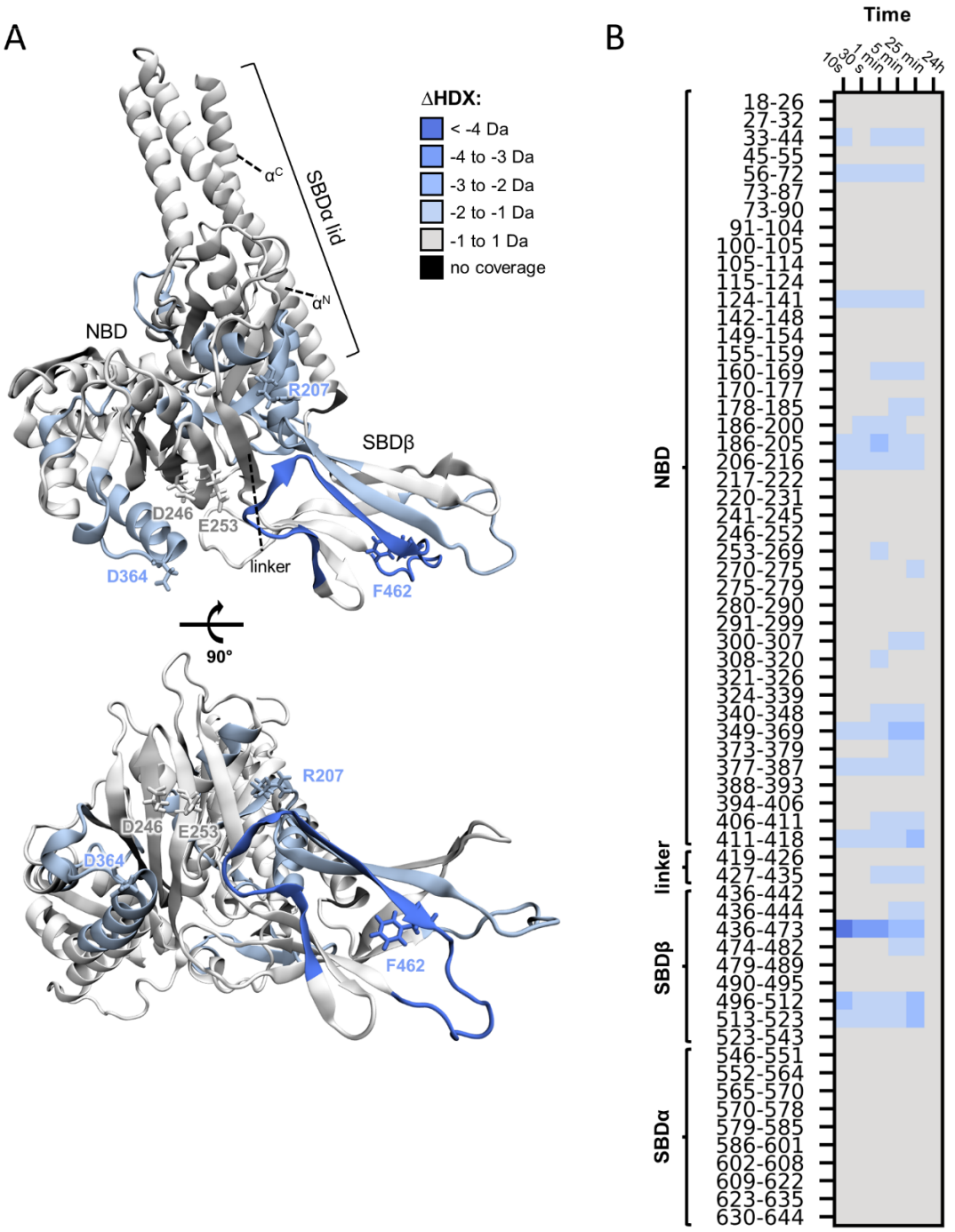
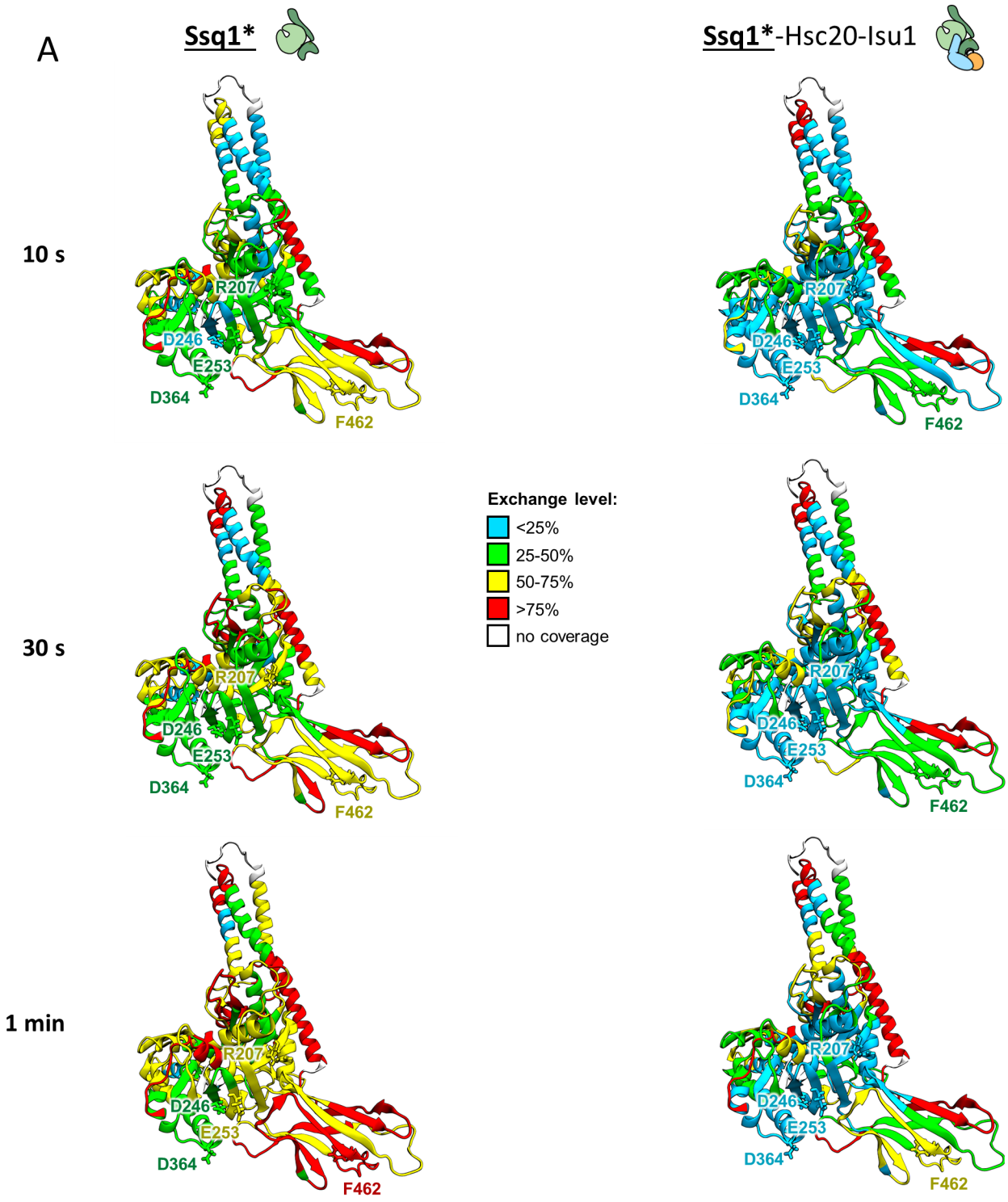


Figure S8 Difference in number of exchanging protons of Ssq1* alone and in the tripartite complex.
(A) Differential deuterium uptake of Ssq1* mapped on the structural model of Ssq1. R207, D246, E253, D364 – residues of the NBD interacting with Hsc20; F462 - residue of the substrate binding cleft of SBDβ interacting with LPPVK of Isu1. Data shown are for 30 s exchange time point.
(B) A heatmap of deuterium uptake difference between Ssq1* in the tripartite complex (Ssq1*-Hsc20-Isu1) and Ssq1* alone. Time points, representative peptides, and domains of Ssq1* are indicated.


A
Ssq1*
Ssq1*-Hsc20-Isu1
10 s
30 s
1 min
R207
D246
E253
D364
F462
Exchange level:
<25%
25-50%
50-75%
>75%
no coverage

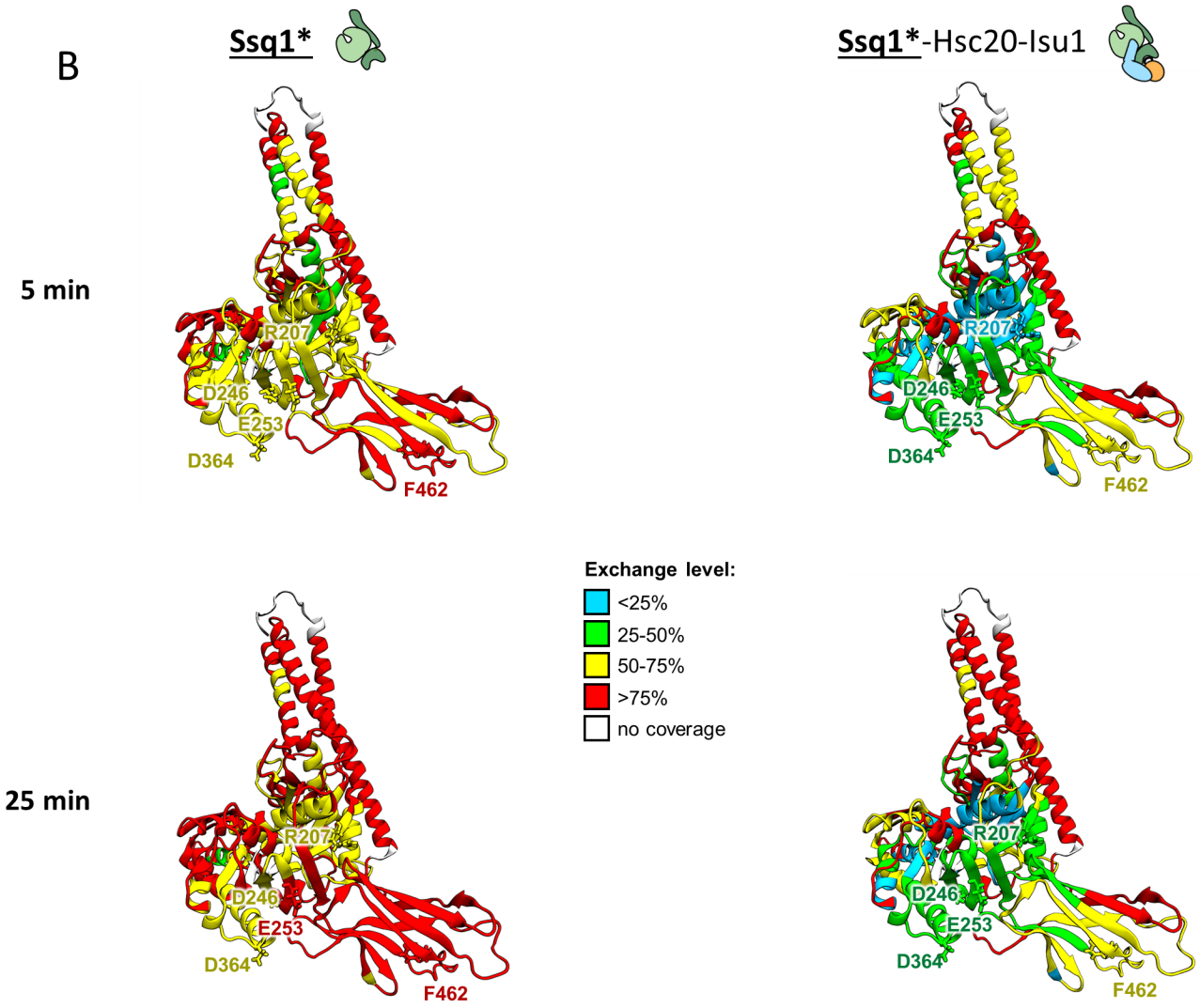


Figure S9. HDX-MS of Ssq1*.
(A, B) Deuterium uptake of Ssq1* alone (left) and in the tripartite Ssq1*-Hsc20-Isu1 complex (right) for different exchange time points. Relative fractional deuterium uptake is mapped on the structural model of Ssq1. Residues involved in interaction with Hsc20 (R207, D246, E253 and D364) and with Isu1 (F462) are indicated.

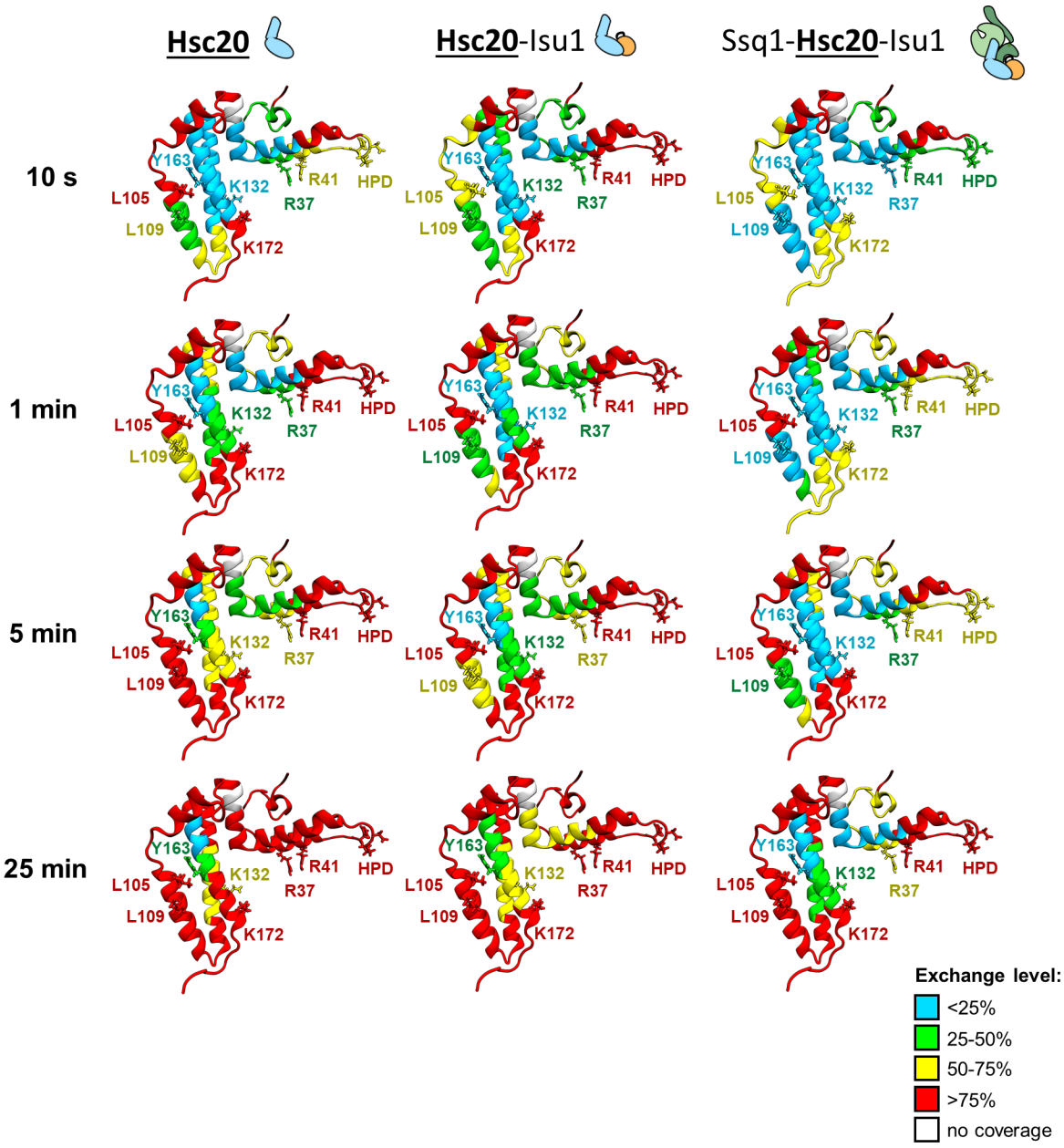


Figure S10. HDX-MS of Hsc20.
Deuterium uptake of Hsc20 alone (left), in the bipartite Hsc20-Isu1 (middle) and in tripartite Ssq1*-Hsc20-Isu1 (right) complexes for different exchange time points. Relative fractional deuterium uptake is mapped on the structure of Hsc20. Residues involved in interaction with Ssq1 (R37, R41, HPD motif, K132 and K172) and with Isu1 (L105, L109, Y163) are indicated.

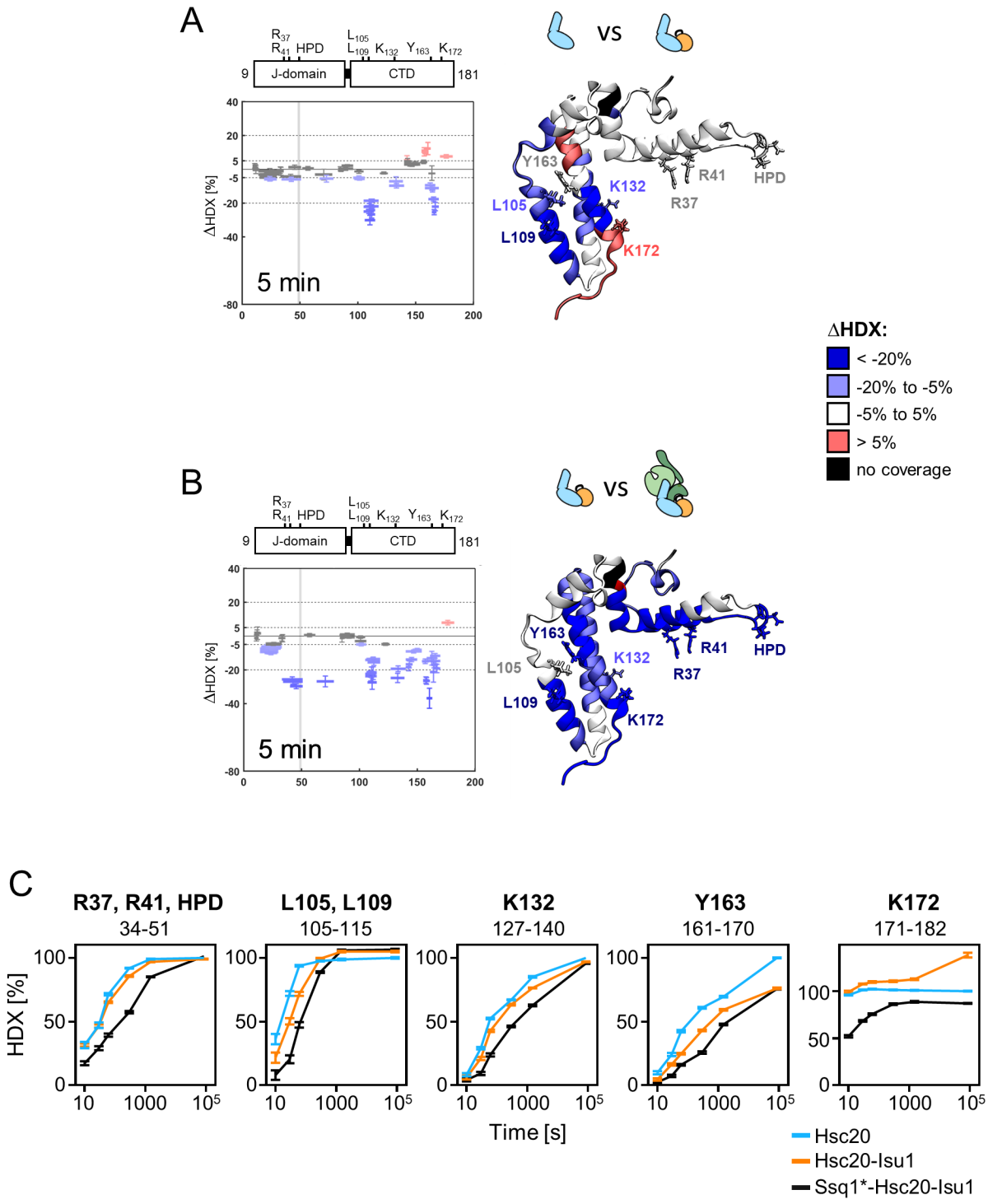


Figure S11. Differential HDX-MS of Hsc20.
(A, B) Difference in deuterium uptake between Hsc20 alone and in the bipartite Hsc20-Isu1 complex **(A)** and between Hsc20 in the bipartite (Hsc20-Isu1) and tripartite (Ssq1*-Hsc20-Isu1) complexes **(B).** Data shown for 5 min exchange time point. (left) Horizontal lines indicate peptides observed; differences in deuterium uptake colored – blue (retarded); red (accelerated). Error bars represent uncertainty in difference in deuterium uptake for a given peptide, as calculated using HaDeX. (right) Differential deuterium uptake mapped on the structure of Hsc20. Residues

involved in interaction with Ssq1 (R37, R41, HPD motif, K132 and K172) and with Isu1 (L105, L109, Y163) are indicated.
(C) Kinetics of relative deuterium uptake into peptides of Hsc20 containing residues involved in protein-protein interactions for Hsc20 alone (blue), in the bipartite (Hsc20-Isu1; orange) and in tripartite (Ssq1*-Hsc20-Isu1; black) complexes.

Figure S12. HDX-MS of Isu1.
Deuterium uptake of Isu1 alone (left), in the bipartite Hsc20-Isu1 (middle) and in tripartite Ssq1*-Hsc20-Isu1 (right) complexes for different exchange time points. Relative fractional deuterium uptake is mapped on the structural model of Isu1. Residues involved in interaction with Ssq1 (PVK 134-136) and with Hsc20 (L63, V72, F94) are indicated.

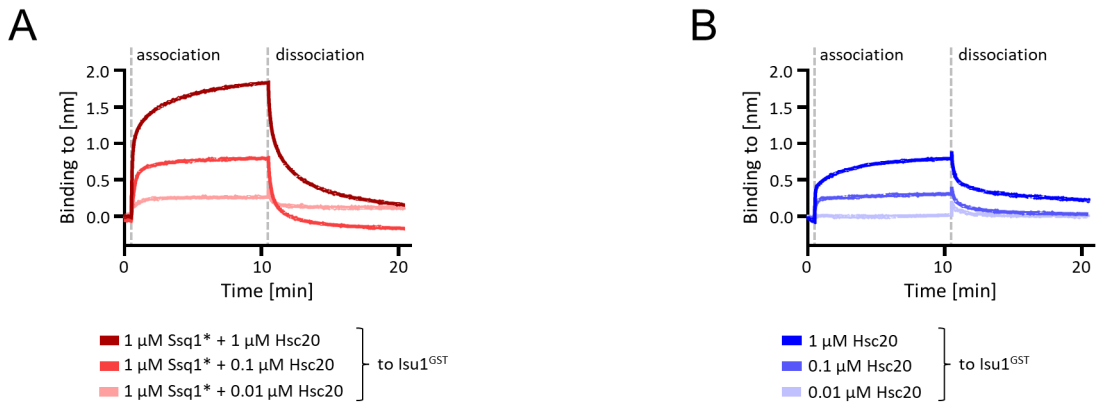


Figure S13. Concentration-dependent kinetics of the tripartite complex formation.
(A, B) BLI sensors were loaded with Isu1^{GST}. Association: loaded sensors were inserted into solutions containing mixture of Ssq1* (1 µM) and Hsc20, at indicated concentrations **(A)** or Hsc20 alone **(B),** as indicated. Dissociation: the sensors were placed into solutions without proteins.

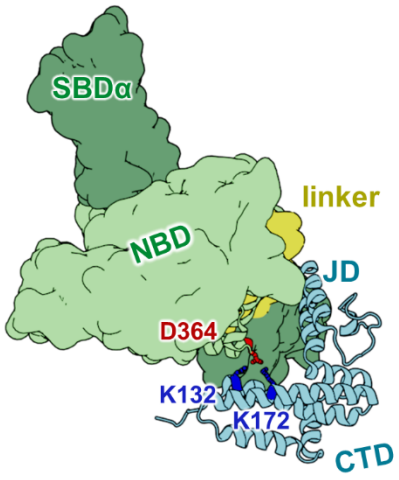


Figure S14. Illustration of the newly identified Hsc20/Ssq1 binding site in the structural model of the Ssq1(ATP)-Hsc20 complex.
The residues forming the new interaction site (K132 and K172) of Hsc20 and (D364) of Ssq1 are in close on the structural model of Hsc20 alone interacting with Ssq1-ATP [6], suggesting that this interaction is Isu1-independent. Hsc20 – light blue, Ssq1: NBD – light green, SBD α and β dark green, interdomain linker- yellow. JD and CTD: J-domain and C-terminal domain of Hsc20, respectively.

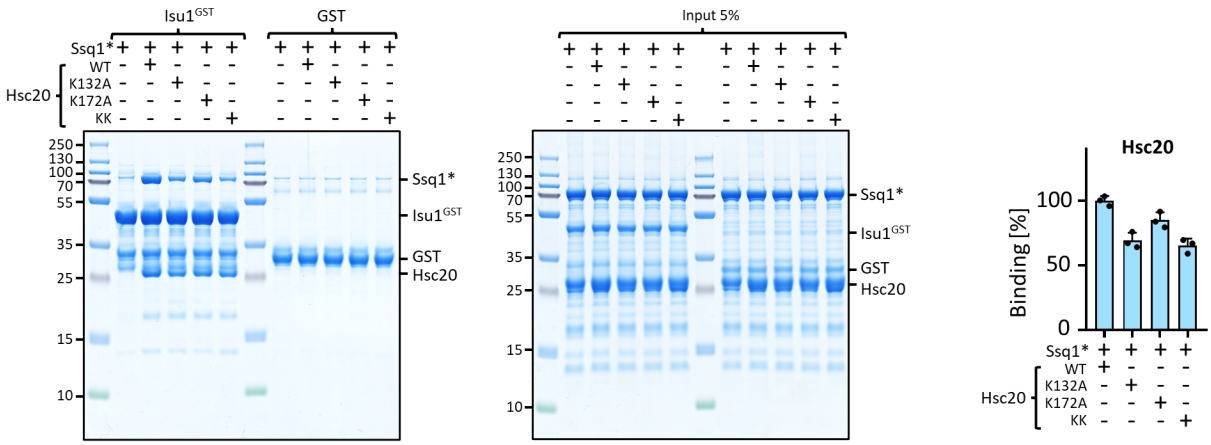


Figure S15. SDS-PAGE gels and quantifications of Ssq1* and Hsc20 for Figure 4. (left) The entire gel for pulldown experiment presented in Figure 4(C). Isu1^{GST} or GST at 2.5 μM was incubated with Ssq1* (5 μM) and Hsc20 (2.5 μM) wild-type (WT) or individual (K132A and K172A) or double (K132A, K172A- KK) substitution variants. Glutathione resin was added to pull-down GST and associated proteins, which were then separated by SDS-PAGE and stained with Coomassie blue. (middle) The loading control gel for pulldown experiment presented in Figure 4(C). "Input 5%" - indicates 5% loading control. (right) Quantification of Hsc20 for three independent experiments. The amounts of pulled down Hsc20 were quantified by densitometry and corrected for background binding to GST alone; protein levels for Isu1^{GST} interacting with Ssq1* and Hsc20 WT were set at 100%.

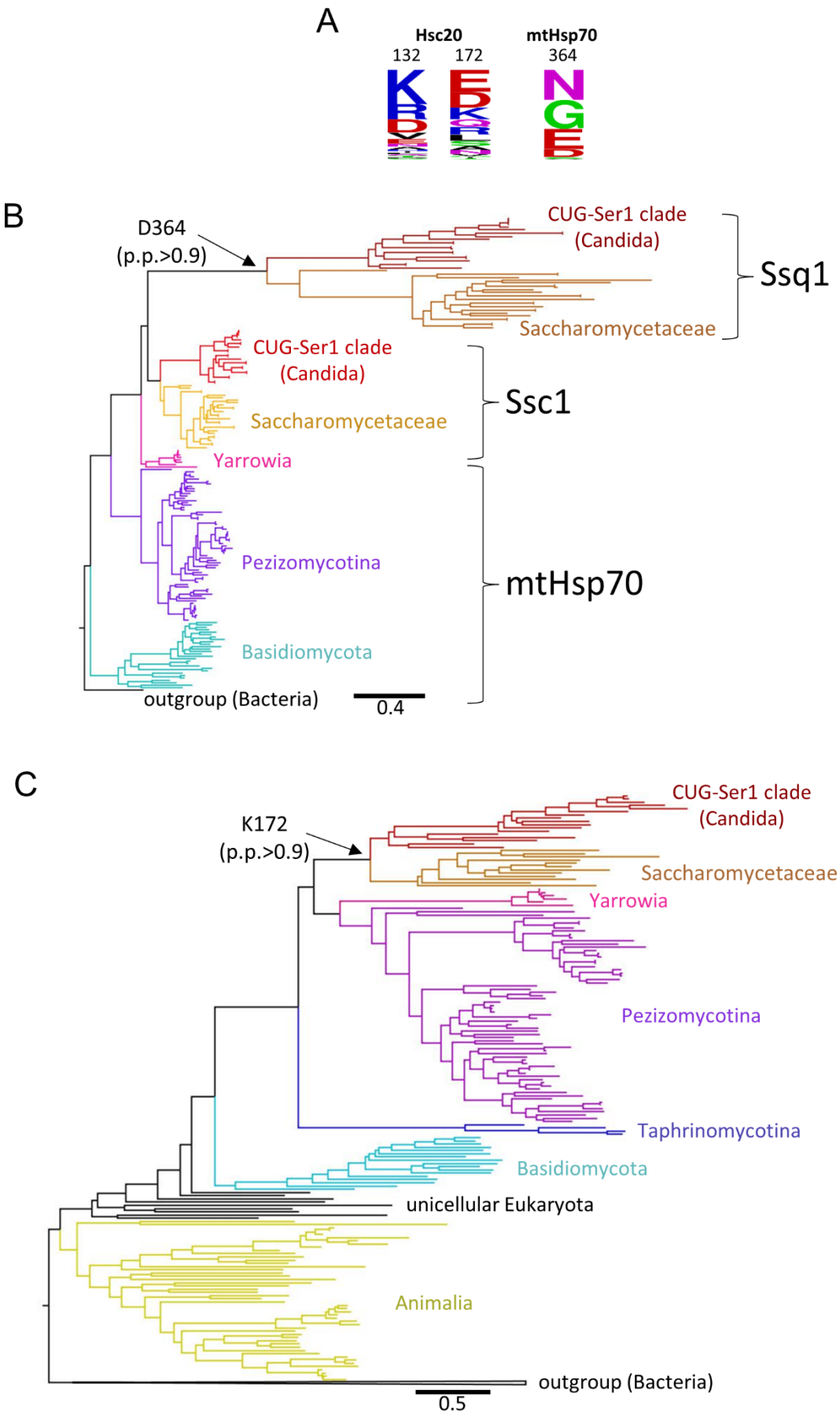

A
Hsc20
132
172
mtHsp70
364
B
D364
(p.p.>0.9)
CUG-Ser1 clade
(Candida)
Saccharomycetaceae
Ssq1
CUG-Ser1 clade
(Candida)
Saccharomycetaceae
Ssc1
Yarrowia
Pezizomycotina
Basidiomycota
mtHsp70
outgroup (Bacteria)
0.4
C
K172
(p.p.>0.9)
CUG-Ser1 clade
(Candida)
Saccharomycetaceae
Yarrowia
Pezizomycotina
Taphrinomycotina
Basidiomycota
unicellular Eukaryota
Animalia
outgroup (Bacteria)
0.5

Figure S16. Evolution of the additional Hsc20-Ssq1 interaction.
(A) Sequence variability at positions of Hsc20 and Ssq1/Ssc1/mtHsp70, which in *S. cerevisiae* are involved in additional Hsc20-Ssq1 interaction. Frequency logos are shown for position 132 and 172 of Hsc20 and 364 of Ssq1/Ssc1/mtHsp70 for orthologs from 12 species of Saccharomycetaceae clade, 12 species of CUG-Ser1 (Candida) clade, 6 species of Yarrowia clade, 63 species of Pezizomycotina clade, 14 species of Basidiomycota clade as well as 41 Animalia and 29 Plantae species.
(B) Maximum likelihood phylogeny of fungi mtHsp70/Ssq1/Ssc1 sequences. A common ancestor of Ssq1 (arrow) had D at position 364 with posterior probability >0.9.
(C) Maximum likelihood phylogeny of Hsc20 from fungal and animal species. A common ancestor of Hsc20 from species in which Hsc20 partners with Ssq1 (arrow) had K at position 172 with posterior probability >0.9. Scale bars: amino acid substitutions per site.